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# Binding of low mobility group protein from rat liver chromatin with histones studied by chemical cross-linking

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The protein of molecular weight about 160 kD (designated LMG160) was isolated from purified low mobility group chromatin proteins. Polyclonal antibody directed against the LMG160 protein in mouse was raised. The specificity of the antibody was determinated with the use of ELISA. Using chemical cross-linking procedure followed by immunoprecipitation with the antiLMG160 antibody complex formation with chromatin proteins was demonstrated. Among the proteins that form complexes with LMG160, histones H3, H2A, and H4 were identified (Western blotting technique).


Nonhistone proteins extracted from chromatin with 0.35 M NaCl can be conveniently fractionated by trichloroacetic acid precipitation into two groups: high mobility group /HMG/ and low mobility group /LMG/ (1,2). The LMG proteins are so defined because of their relative mobilities upon electrophoresis in acid-urea polyacrylamide gels (1,2). The LMG proteins are abundant and heterogenous class of loosely bound nonhistone chromatin proteins which were studied far less intensively than any other nonhistone proteins (3-10).Previous investigations from our lab have shown that the LMG proteins are more likely to be structural proteins because they are present in chromatin in large amounts. However, simultaneously we have shown that some of LMG proteins are bound to the DNase II-sensitive

ABBREVIATIONS
LMG - low mobility group; HMG - high mobility group; SDS -sodium dodecyl sulphate; PMSF - phenyl-methyl-sulphonyl fluoride; DSP - dithiobis/succinimidyl propionate/; DMF - dimethylformamid; BSA - bovine serum albumin.

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fraction of chromatin (9,11) and may affect transcription in vitro (10).

One of the major obstacles in examining the LMG proteins to determine their function/s/ is their heterogeneity. Thus,as a preliminary step, it is necessary to fractionate them in order to obtain more homogenous group. We believe that only a homogenous preparation of particular LMG proteins will aid the ongoing research to determine their biological role.

In the present paper we describe a single LMG fraction, designated LMG160, isolated from the 0.35 M NaCl extract of rat liver chromatin and generation of polyclonal antibody to LMG160. This antibody has allowed us to investigate the interaction of LMG160 with chromatin proteins detected by chemical cross-linking followed by SDS-polyacrylamide gel electrophoresis. The results suggest that LMG160 interact with only a few nonhistone chromatin proteins and histones H3,H2A and H4.

## MATERIALS and METHODS

The subsequent procedures were performed at $4^{o}C$ in the presence of 2 mM PMSF as an inhibitor of proteases.

Isolation of LMG proteins. Nuclei were isolated from rat liver as described elsewhere (12). Rat liver nuclei were centrifuged through 2.2 M sucrose in 0.01 M Tris-HCl /pH 7.8/, 0.003 M MgCl . Chromatin was obtained by the procedure described elsewhere (13): nuclear pellet was washed with 0.14 M NaCl, 0.01 M Tris-HCl /pH 8.3/ and centrifuged through 1.7 M sucrose, 0.01 M Tris-HCl /pH 7.9/. In the next step LMG proteins were isolated with the minor modifications as described elsewhere (2,8) and analyzed by SDS-PAGE according to the method of Laemmli (14).

Production of antibody to LMG proteins. LMG proteins disolved in PBS were emulsified in an equal volume of complete Freund's

adjuvant and injected subcutaneously into one rabbit (1.5 mg per rabbit). In subsequent two injections /intra joints/ instead of complete incomplete adjuvant was used. After third injection blood was drawn and the obtained antiserum was analysed by ELISA according to (15) to identify the specificity of antiserum to LMG proteins. The antibody titer was 2048.

Purification of LMG proteins by immunoaffinity chromatography. LMG proteins,solubilized in 0.1 M $NaHCO_3$, 0.5 M NaCl /pH 8.5/ (1mg/1ml) were loaded onto a column packed with 5 ml of CNBr-activated Sepharose 4B that had been stabilized by glutaraldehyde crosslinking (16). The load recirculated through the column for 12 houres at a flow rate of 0.7 ml/min. The column was washed with 50 volumes of 0.05 M Tris-HCl /pH 7.2/, 0.15 M NaCl, 0.1% SDS,1% Na deoxycholate, 1% Triton X-100 and with 50 volumes of 0.01 M Tris-HCl /pH 7.8/, 0,001 M $CaCl_2$ . The LMG proteins were eluted with 50% ethylene glycol /pH 11.5/ (17).
Eluted LMG proteins were subsequently precipitated with abso-lute ethanol at $-20^{\circ}$ C overnight. The precipitate was extracted with acetone before resolving by SDS-PAGE according to Laemmli (14).

Isolation of LMG160 protein. Purified by immunoaffinity chromatography LMG protein fractions were separated on an SDS/10% polyacryloamide slab gel /0.75 mm thick/ using the buffer system of Laemmli (14). Subsequently the LMG protein fraction of molecular weight about 160 kD (named LMG160) which was identified by soaking the gel in cold 1 M KCl, was eluted from the gel slices in electroelutor /model UEA, IBI/ and dialysed to PBS or sample buffer.

Production of antibody to LMH160 protein. The LMG160 protein disolved in PBS (25 $\mu$/ 0.1 ml) was emulsified in an equal volume of complete Freund's adjuvant and injected intraperitoneally

into a Balb/c mouse. The mouse was then boosted four times at two weeks intervals. After the fourth injection /without adjuvant/ blood was withdrawn. The obtained antiserum was analysed by ELISA according to (15) to identify the specificity of the LMG160 antiserum. The antibody titer was at least 2048.

Chemical cross-linking. Cross-linking was performed in buffers devoid of Tris, EDTA and 2-mercaptoethanol. Fresh whole nuclei from rat liver were dissolved in 0.01 M sodium phosphate /pH 7.4/, 0.25 M sucrose, 0.003 M $MgCl_2$. The concentration of chromatin was adjusted to 0.5 mg DNA/ml. Cross-linking reaction was performed at 0°C with DSP freshly dissolved in DMF (0.5 µmole/ 1 mg of chromatin) (18). After 5 minutes cross-linking reaction was terminated by addition of 160 µmoles of chilled sodium acetate. Then, the solution was dialysed against 0.025 M Tris-HCl /pH 7.3/, 0.005 M EDTA, 0.15 M NaCl, 0.5% sodium deoxycholate, 0.05% SDS overnight at 12°C. Fresh buffer was replaced three times. Solution was dialysed against 0.1 M Tris-HCl /pH 7.5/, 0.05 M NaCl, 0.005 M $MgCl_2$, then centrifuged at 100 000 g for 45 minutes. After centrifugation the pellet was rejected and supernatant S1 was adjusted to 0.25% BSA and used for immunoprecipitation reaction.

Immunoprecipitation of LMG160-cross-linked products. Cross-linked complexes between LMG160 and the other chromatin proteins were immunoprecipitated according to procedure described by Dorbic et al. (19) with some modifications. Supernatant S1 was incubated with LMG160-antiserum and parallely with control antiserum from nonimmunized mice, followed by incubation with PANSORBIN /formaline-fixed Staphyloccocus aureus cells,Calbiochem/ for 3 houres at 4°C and pelleted in Microfuge at 15 000 g for 2 minutes. The pellet was washed twice with PBS enriched with 0.005 M EDTA, 0.25% BSA. The proteins were dissolved 3 minutes at 95°C

with 0.0625 M Tris-HCl /pH 6.8/, 2% SDS, 0.002 M EDTA, 30% glicerol. After centrifugation supernatant designated S2 was analysed by SDS/10% polyacrylamide slab gel (14). Gels were stained by silver and Coomassie blue procedures (20).

Radiolabeling of proteins. Protein A and marker proteins were iodinated using IODO-BEADS Iodination Reagent /Pierce/.

Western blotting. Western blots were performed based on the technique developed by Towbin, et al. (21). Final concentration of labeled second antibody /protein A/ was 2 x $10^5$dpm/ml.

Concentrations of proteins and DNA. The concentration of chromatin proteins was determined by assuming: $E_1^{280}$ mg/ml = 0.82 (22) and the concentration of DNA was determined using the conversion 50$\mu$g/A.

## RESULTS

Since the attempts to fractionate the LMG proteins reported in literature (4,5,7) did not result in obtaining homogenous proteins it was decided to purify the protein by affinity chromatography. The anti-LMG antibody was raised in rabbits and the titer determined by ELISA was 2048 /not shown/. The antibody was coupled to CNBr-Sepharose 4B, and cross-linked with glutaraldehyde in order to forcing binding the antibody with the matrix.

Chromatography with the use of the Sepharose 4B affinity column followed by polyacrylamide gel electrophoresis revealed 5 protein fractions /Fig.1,lane 2/ of mol.wt. ranging from 35 - 200 kD. The proteins eluted from the affinity column were the highly immunogenic ones. The affinity purified LMG protein of mol.wt. about 160 kD was electroeluted from the gel. PAGE revealed a single band of mol.wt. about 160 kD designated LMG160 /Fig.1,lane 3/.

The pI determined by isoelectrofocusing (23) was below 7 /not shown/. With the use of this electrophoretically homogeneous protein an antibody was produced in Balb/c mice. The resulting antiserum had a titer of 2048 and did not react with BSA or rat liver histones when tested by ELISA /not shown/.

Cross-linking experiments.

The cross-linking reaction with DSP was performed on morphologically intact nuclei isolated from rat liver. This allowed to maintain the native structure of chromatin. The DSP bound proteins remain in intact complexes during the consecutive stages

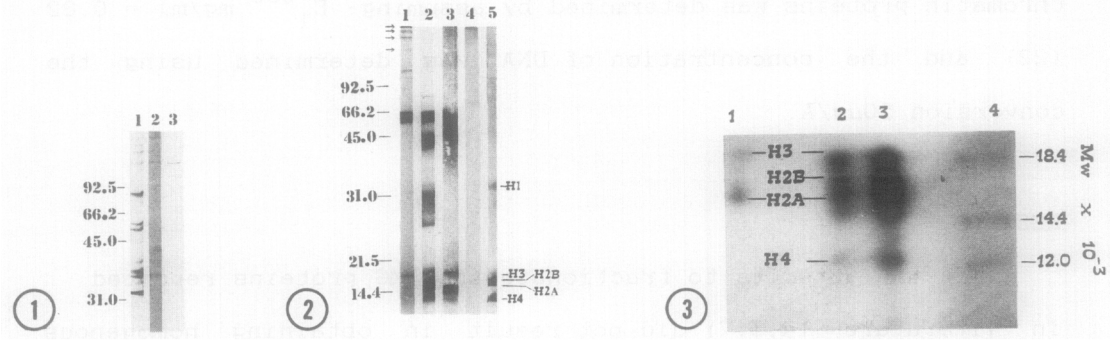


Fig.1 Silver staining after electrophoresis on 10% polyacrylamide gel of: (1) rat liver LMG proteins /50 μg/, (2) LMG protein fractions purified by immunoaffinity chromatography /20 μg/, (3) LMG160 protein /2 μg/. The positions of mol. wt. standards are indicated by bars /92 500 - phosphorylase B, 66 200 - BSA, 45 000 - ovalbumin, 31 000 - carbonic anhydrase, 21 500 - soybean trypsin inhibitor, 14 400 - lysozyme/.

Fig.2 Silver staining after electrophoresis on 10% polyacrylamide gel of: (1) complexes of chromatin proteins formed by cross-linking with DSP and immunoprecipitated with LMG160-antiserum /50μg/ , (2) chromatin proteins from cross-linking complexes /lane 1/ in the presence of 5% 2-mercaptoethanol /50 μg/, (3) Chromatin proteins from cross-linking complexes which on lane 1 formed the second band in the presence of 5% 2-mercaptoethanol, (4) Control immunoprecipitation of chromatin proteins with non-specific serum from nonimmunized mice /in the presence of 5% 2-mercaptoethanol/, (5) Histones from rat liver chromatin /10 μg/.

Fig.3 Autoradiography after Western blot and histones-antiserum detection: (1) Chromatin proteins from cross-linking complexes in the presence of 5% 2-mercaptoethanol, (2) Histones from rat liver chromatin /100 μg/, (3) Histones from rat liver chromatin /300 μg/, (4) The $^{125}$I-labeled mol.wt. standards /18 400 - β-lactoglobulin, 14 400 - lysozyme, 12 000 - cytochrome c/.

of chromatin isolation. The complexes that contain LMG160 protein were obtained by immunoprecipitation with anti-LMG160 antiserum followed by PAGE /designated by arrows in Fig.2, lane 1/. The remaining bands of mol. wt. below 160 kD represent proteins released from the native complexes by sulphydryl reagents. In order to compare directly the preparations, the samples incubated for 24 houres with 2-mercaptoethanol were separated on the same gel. These proteins might constitute, at least in part, contaminants generated unspecific during immunoprecipitation reaction /e.g.heavy and light chains of immunoglobulins/ although the immunoprecipitation with the use of control serum of nonimmunized mice /50 μg of S1 supernatant / contained only tracer amount of the antibodies /Fig.2, lane 4/.

The immunocomplexes precipitated with LMG160 anti-serum were destroyed upon 24 houres incubation with 2-mercaptoethanol. This allowed to isolate by PAGE proteins that formed complexes with LMG160 protein /Fig.2, lane 2/. Fig.2 lane 3 represents chromatin proteins that form second band from the top /Fig.2, lane 1/. This band was cut out of the gel, electroeluted and incubated for 24 houres with sample buffer containing 2-mercaptoethanol. The reaction mixture was applied to the stacking gel and subjected to PAGE. The dark background observed in the gels results from the incubation of the samples with 2-mercaptoethanol /5%/ prior to silver staining. As seen in Fig. 2 lane 3, proteins of mol.wt. ranging from 31 to 25 kD were not detected.

Identification of the proteins that formed cross-linked complexes.

At the present stage of the investigations, because of lack of the appropriate antisera, it was possible to identify only the proteins of mol.wt. below 20 kD. Based on characteristic band pattern these proteins could be identified as histones. In order

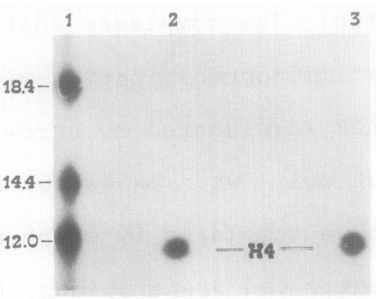


Fig.4 Autoradiography after Western blot and histone H4-antiserum detection: (1) The $^{125}$I-labeled mol. wt. standards /18 400- β-lactoglobulin, 14 400 - lysozyme, 12 000 - cytochrome c/, (2) Chromatin proteins from cross-linking complexes in the presence of 5% 2-mercaptoethanol /300 μg/, (3) Histone H4 /100 μg/.

to verify this possibility the histones were isolated from rat liver /Fig.2,lane 5/ and mice anti-histone serum was obtained. Fig. 3 represents Western blot following electrophoretic separation of chromatin proteins that form cross-linked complexes with LMG160 protein in the presence of 5% 2-mercaptoethanol /Fig.3,lane 1/ and also of rat liver histones /Fig.3,lane 2 and 3/. The blots were reacted with anti-histone serum and freshly iodinated protein A as a second antibody. This was followed by autoradiography. The immunoreactive bands in Fig.3,lane 1 allowed to identify histones H3 and H2A among the proteins that formed complexes with LMG160 protein. Because of the observation made on the weakest reaction of histone H4 with histone antiserum /Fig.3, compare line 2 with 3/ a succesive Western blot was made and histone H4 antiserum was used for detection of protein. As shown in Fig.4 this procedure allowed to identify histone H4 among the proteins that form complexes with LMG160 protein.

## DISCUSSION

The functions of the LMG proteins are largely unknown, alike the role of majority of the nonhistone chromatin proteins other than these having recognized enzymatic activities. The LMG proteins are believed rather to be structural component of chromatin

because of their relatively high concentration (4,8). Most of the LMG proteins are tissue unspecific, although there exist minor cell specific differences, both qualitative and quantitative, as evidenced by the gel patterns /data not shown/. It seems that one of the major importance in elucidating the biological role of LMG proteins is to determine specific interactions between these proteins and other chromatin proteins. However this must be preceeded by isolation of a single LMG species. The interactions between chromatin proteins play an important role in maintaining the chromatin structure and probably also its function. Moreover the presence of both LMG proteins and histones in chromatin in relatively high concentration strongly suggest that these two types of proteins interact.

Present report describes the method of isolation by electroelution of a single LMG protein species, designated LMG160 of mol.wt. of about 160 kD and pI below 7.The isolation of this protein was preceeded by purification by immunoaffinity chromatography of the LMG proteins yielding a final preparation comprising only five fractions /Fig.1/. Obtaining a single protein species allowed to raise a polyclonal antibody directed against the LMG160 protein. The specificity of the antibody was verified by the use of ELISA.When this test was carried out using the same concentration of LMG160 protein, rat liver histones and bovine serum albumin /BSA/, no reactivity was observed with histones and BSA /data not shown/. This allowed us to prepare the cross-linked complexes by an immunological approach. In order to obtain the protein complexes we have chosen DSP, a readily cleavable cross-linking reagent which reacts with free amino groups and does not affect the structure of chromatin (18). The reversibility of the cross-linking reaction upon sulphydryl reagents allowed to isolate the cross-linked proteins in purified form.

The cross-linking results gave information that there was a cross-linking of LMG160 protein with a few nonhistone proteins /imposible to identify at this stage/, and to those which could be identified as histones /Fig.2,lane 2 and 3/ on the basis of characteristic pattern of protein bands revealed by silver staining. Western blotting followed by reaction with a polyclonal anti-histone serum allowed to identify histones H3 and H2A in the cross-linking material /Fig.3,lane1/. However, the reactivity of the anti-serum with histone H4 was the weakest of all histones /Fig.3,lane2/. Therefore we decided to use anti-H4 polyclonal antibody to test whether the H4 histones are really absent from the complex or else whether the results presented in Fig.3 were simply due to the low reactivity of anti-serum against histone H4 rather than to its absence in the complex.

The experiment with the use of polyclonal antibody directed against histone H4 allowed to identify also histone H4 among the proteins that form cross-linked complexes with LMG160 protein /Fig.4/.

No reaction with the antibodies directed against histone H1 or histone H2B was performed yet. Therefore the contribution of histones H1 or H2B in formation of the complexes with LMG160 protein could not be excluded.

Although the results clearly indicate the interaction of LMG160 protein with histones H3, H2A and H4, it could not be concluded whether the interactions are specific and what is the relation of the structure of LMG160 protein to complex formation. Because of the efficiency of interaction of LMG160 protein with histones /H3,H2A,H4/ it might be expected that the LMG160 protein interact with all or almost all histones in all chromatin regions. The precise location of the LMG160 protein in chromatin structure remains to be elucidated.Among

other techniques the investigations on the association of LMG160 protein with mononucleosomes of transcriptionally active as well as inactive chromatin might be useful in solving the problem of the role of the complex formation in the structure and function of chromatin.

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